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SOSTDC1 differentially modulates Smad and beta-catenin activation and is down-regulated in breast cancer

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Abstract

Sclerostin domain containing 1 (SOSTDC1) protein regulates processes from development to cancer by modulating activity of bone morphogenetic protein (BMP) and wingless/int (Wnt) signaling pathways. As dysregulation of both BMP and Wnt signaling has been observed in breast cancer, we investigated whether disruption of SOSTDC1 signaling occurs in breast cancer. SOSTDC1 mRNA expression levels in breast tissue were examined using a dot blot. Affymetrix microarray data on SOSTDC1 levels were correlated with breast cancer patient survival using Kaplan–Meier plots. Correlations between SOSTDC1 protein levels and clinical parameters were assessed by immunohistochemistry of a breast cancer tissue microarray. SOSTDC1 secretion and BMP and Wnt signaling were investigated using immunoblotting. We found that SOSTDC1 is expressed in normal breast tissue and this expression is reduced in breast cancer. High levels of SOSTDC1 mRNA correlated with increased patient survival; conversely, SOSTDC1 protein levels decreased as tumor size and disease stage increased. Treatment of breast cancer cells with recombinant SOSTDC1 or Wise, a SOSTDC1 orthologue, demonstrated that SOSTDC1 selectively blocks BMP-7-induced Smad phosphorylation without diminishing BMP-2 or Wnt3a-induced signaling. In conclusion, SOSTDC1 mRNA and protein are reduced in breast cancer. High SOSTDC1 mRNA levels correlate with increased distant metastasis-free survival in breast cancer patients. SOSTDC1 differentially affects Wnt3a, BMP-2, and BMP-7 signaling in breast cancer cells. These results identify SOSTDC1 as a clinically important extracellular regulator of multiple signaling pathways in breast cancer.

Keywords

Beta-catenin (BMP); Bone morphogenetic protein(BMP); Breast cancer; Sclerostin domain containing 1 (SOSTDC1); Wingless/int (Wnt); Wise

Introduction

Despite significant advances in prevention, diagnosis, and treatment, breast cancer remains the second most deadly cancer in women in the United States [1]. Treatment of breast cancer is complicated by the distinct subtypes that exist, each with a unique disease course and optimal treatment mode [2–4]. These disparities highlight the need for new therapeutic and diagnostic targets for breast cancer. Potential sources for such targets are the molecular signaling pathways commonly disrupted in cancer cells [5–9], especially those that regulate cancer-relevant cellular functions such as cell cycle progression, cellular proliferation, and cell death.

Sclerostin domain containing 1 protein (SOSTDC1) is a critical regulator of cell signaling, participating in processes from development to cancer. Highly conserved within vertebrates, orthologues of SOSTDC1 have been identified in species from *Xenopus* to humans [10].

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SOSTDC1 has been implicated in a range of normal processes including developmental patterning [11], tooth development [12], hair growth [13], and reproduction [14]. SOSTDC1 also impacts disease processes such as renal injury [15], adult renal cancer [16], and pediatric Wilms' tumors, where its gene has been identified as a candidate tumor suppressor gene [17].

One way that SOSTDC1 affects these diverse processes by regulating the activity of bone morphogenetic proteins (BMPs, [18, 19]). BMPs are ligands that activate pathways involved in cell proliferation and differentiation. Binding of BMP proteins to BMP receptors leads to receptor phosphorylation [20, 21]. Phosphorylated BMP receptors recruit and phosphorylate receptor-regulated Smad proteins, Smad-1, -5, and -8. Phosphorylated receptor Smads associate with Smad-4, which permits nuclear translocation of the Smad complex. In the nucleus, this complex induces the transcription of cell regulatory factors including *p53, p21,* and *Bcl-2-associated X* (*bax*; reviewed in [22, 23]). SOSTDC1 inhibits BMP signaling by binding directly to select BMP proteins including BMP-2, -4, and -7 [24]. This binding blocks the interaction of BMPs with their receptors, thus limiting BMP activity.

SOSTDC1 can also regulate the wingless/int (Wnt) signaling pathway. Activation of the Wnt signaling pathway centers around the inactivation of an inhibitory complex that includes Axin, adenomatous polyposis coli protein (APC), and beta-catenin. Prior to Wnt pathway activation, the protein kinases Glycogen Synthase Kinase-3 (GSK-3) and casein kinase 1 gamma (CK1gamma) promote the phosphorylation of beta-catenin and APC. These phosphorylation events target beta-catenin for ubiquitination and proteosomal degradation. Transition to the active state is induced by the binding of a Wnt ligand to the Frizzled (Fzd) and low density lipoprotein receptor-related protein (LRP) receptor complex, promoting phosphorylation of LRP and other signaling proteins. Phosphorylated LRP can then associate with Axin, releasing beta-catenin from the inhibitory complex. Dissociation of beta-catenin prevents its phosphorylation, ubiquitination, and degradation. Stabilized beta-catenin then transits to the nucleus where it promotes transcription of Wnt pathway targets (reviewed in [25]). These targets are situation dependent, varying between cell types and activation contexts. Two cancer-relevant targets include c-Myc and cyclin D1 [26–28].

SOSTDC1 can either enhance or inhibit Wnt signaling. For example, SOSTDC1 decreases Wnt signaling by impeding the binding of Wnt8 to the LRP6 receptor [11]. The localization of SOSTDC1 also affects its activity: secreted SOSTDC1 can have either inhibitory or activating effects, but SOSTDC1 within the endoplasmic reticulum (ER) is exclusively inhibitory [29]. The capability of SOSTDC1 to mediate distinct effects on the pathway makes it a versatile Wnt pathway modulator.

Dysregulation of both BMP and Wnt signaling has been observed in breast cancer [30–36], suggesting that SOSTDC1, through its ability to modulate one or both of these pathways, may contribute to breast cancer development or progress. Indeed, recent results from our lab have demonstrated that SOSTDC1 expression is reduced in renal cell carcinoma [16]. In this context, SOSTDC1 leads to decreased cellular proliferation and inhibition of Wnt3a- and BMP-7-induced signaling [16].

We tested the hypotheses that SOSTDC1 levels were decreased in breast cancer and that SOSTDC1 affected Wnt- and BMP-induced signaling in breast cancer cells. We found that SOSTDC1 mRNA and protein levels were reduced in breast cancer patient samples, and that SOSTDC1 levels correlated with patient outcomes. Studies of cellular signaling showed that the addition of exogenous SOSTDC1 and its orthologue, Wise, affected signaling induced by Wnt3a, BMP-2, and BMP-7. Wise cotreatment led to modestly increased Wnt3a-induced beta-catenin stabilization, decreased BMP-7-mediated Smad phosphorylation, and did not

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affect BMP-2 signaling. The complex signaling activity of SOSTDC1 in breast cells and its disruption in breast cancer suggests that this regulatory axis may contribute to breast cancer development.

Materials and methods

Cancer profiling array

The Cancer Profiling Array I (BD Clontech) was probed with radiolabelled SOSTDC1 per manufacturer's instructions. Resulting hybridization signals were scanned and quantified with UnScanIt software (Silk Scientific). Negative controls, including yeast total RNA, yeast tRNA, and *E. coli* DNA, did not hybridize with the SOSTDC1 probe.

Gene expression and survival analysis

SOSTDC1 gene expression in primary human breast tumors were examined by microarray analysis in a super cohort of 759 primary breast cancers derived from six independent breast cancer cohorts [37–41], as shown in Supplemental Table 1. All tumor samples were profiled on the Affymetrix U133 series microarray platforms. Of 759 cases, 741 had corresponding survival data with distant metastasis-free survival (DMFS) as the clinical endpoint. All microarray data were processed using the MAS5.0 algorithm (Affymetrix), scaled to a mean target signal intensity of 500, and $\log_2$ transformed. Using the probe set corresponding to the SOSTDC1 transcript (213456_at), breast cancer cases were ranked by SOSTDC1 transcript levels into gene expression-based quartiles [1st quartile (lowest 25%), $n = 185$; 2nd quartile, $n = 185$; 3rd quartile, $n = 185$, and 4th quartile (highest 25%), $n = 186$]. The DMFS of patients comprising each quartile was assessed by Kaplan–Meier analysis (Sigma Plot 11.0). The significance of hazard ratios was ascertained by the likelihood ratio test p-value.

Tissue microarrays and immunohistochemistry

The tissue microarrays of tumor cores from breast cancer patients with recurring tumors have been described [42]. The blocks were sectioned and fixed on slides. Each section was deparaffinized with xylene, rehydrated, and treated with 0.3% hydrogen peroxide and antigen unmasking solution (AUS, Jackson Labs). After blocking in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), slides were incubated with anti-SOSTDC1 antiserum in blocking solution, followed by treatment with horseradish peroxidase (HRP)-conjugated anti-rabbit Immunoglobulin G (IgG) antibody. Diaminobenzidine (DAB) substrate was used for detection with subsequent hematoxylin counterstaining. Bright field digital images were obtained under equal exposure and magnification conditions. The intracellular staining intensities of these images were quantified as previously reported [43]. Descriptive statistics, including means and standard deviations for continuous measures and frequencies and proportions for categorical variables, were calculated. Spearman's correlation coefficient was used to assess the strength of the relationship between SOSTDC1 and other study variables. The chi-square approximation of the log-rank test was used to test for survival differences between groups defined at different SOSTDC1 cutpoints; proportional hazards regression was utilized in testing the effect of SOSTDC1 univariately on overall and disease-free survival.

Recombinant proteins and reagents

Recombinant human BMP-2, BMP-7, and Dickkopf-1 (DKK1) and recombinant mouse Noggin and Wnt3a were purchased from R&D Systems. The following commercial antibodies were obtained: rabbit polyclonal anti-phospho-Smad 1/5/8 (Cell Signaling Technologies); rabbit polyclonal anti-total Smad 1/5/8 (sc-6031-R; Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH, Fitzgerald); purified mouse monoclonal anti-active-beta-catenin (Millipore); purified mouse monoclonal anti-beta-catenin (BD Biosciences); HRP-conjugated goat anti-rabbit IgG (BioRad); and goat anti-mouse IgG-HRP (BioRad). The generation of anti-SOSTDC1 antiserum and the rhSOSTDC1 mammalian expression vectors have been described [16]. The Wise expression construct was a kind gift from Nobue Itasaki. Wise conditioned media was prepared as previously described [44].

Cells and culture conditions

The mammary ductal carcinoma cell line T47D (HTB-133) and the epithelial adenocarcinoma cell lines MCF7 (HTB-22) and MDA-MB-231 (HTB-26) were maintained as recommended by American Type Culture Collection (ATCC). Human mammary epithelial cells (HMEs; Cambrex Corporation) were maintained in mammary epithelial cell growth media (MEGM; Lonza Walkersville, Inc.). The HME stepwise-transformed cell lines were a gift from the lab of R. Weinberg and were maintained as previously described [45].

Detection of secreted SOSTDC1

Cell lines were weaned to a defined media system over 2 weeks. Cells were seeded at equal densities and were maintained in serum-free media for 72 h. Conditioned media were then removed, filtered, and snap-frozen. Media were concentrated using Amicon and Microcon 10,000 kilodalton (kDa) cutoff spin filters (Millipore). Media samples containing equal amounts of protein were gel-resolved, transferred to nitrocellulose, and probed with SOSTDC1-specific antiserum.

Signaling assays

For BMP and Wnt signaling assays in MDA-MB-231 cells, cells were plated in growth media. Eighteen hours later, cells were serum-starved in Dulbecco's Modified Eagle's Medium (DMEM) with 0.5% fetal bovine serum (FBS) for 48 h. They were then treated with BMP-2 or -7 (40 ng/ml) with or without Noggin (150 ng/ml) or Wise-containing conditioned media. Alternatively, serum-starved cells were treated with Wnt3a (50 ng/ml) in the presence or absence of Dickkopf-1 (DKK1; 100 ng/ml), Wise-containing conditioned media, or control conditioned media. Two hours later, cells were harvested in Triton X-100 lysis buffer [50 mM Tris pH 7.5, 150 mM sodium chloride, 0.5% Triton X-100 (Sigma)] supplemented with Complete protease and phosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostics). Proteins were gel-resolved, transferred to nitrocellulose or polyvinylidene fluoride (PVDF), and immunoblotted. Signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) with the LAS-3000 imaging system (Fujifilm). Immunoblot signals were quantified using Adobe Photoshop CS3 (Adobe Systems).

MCF7 cells were plated in growth media, allowed to attach overnight, and treated with DMEM/F12 with or without BMP-2 or BMP-7 (75 ng/ml) in the presence or absence of Noggin (150 ng/ml) or recombinant human SOSTDC1 (150 ng/ml) for 4 h. Cells were harvested and immunoblotted as previously described [16].

Results

SOSTDC1 message is reduced in breast tumors

SOSTDC1 regulates breast cancer-relevant signaling pathways [11, 24, 29]. Since levels of SOSTDC1 are downregulated in renal carcinoma [16], we tested whether SOSTDC1 was similarly affected in breast cancer. SOSTDC1 mRNA levels were assessed in clinical samples using a radiolabelled SOSTDC1-specific oligonucleotide to probe a cDNA dot blot containing matched normal and tumor samples from breast cancer patients. Quantification

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demonstrated that SOSTDC1 message levels were significantly lower in the tumors than in the matched normal tissue in 47 of the 53 sample pairs (89%; Fig. 1). Overall, SOSTDC1 message levels in tumors averaged approximately 60% that in normal tissue ($P < 0.05$, Fig. 1). A further reduction in SOSTDC1 was observed when mRNA from distant metastases ($n = 3$) and that from normal breast tissue ($n = 50$) were compared ($P < 0.01$, Fig. 1). These findings demonstrate that, like in renal carcinoma, SOSTDC1 mRNA levels are reduced in breast tumors and metastases relative to normal breast tissue.

High SOSTDC1 message levels correlate with increased distant metastasis-free survival

We then asked how SOSTDC1 expression is distributed in a large population of primary invasive breast cancers and whether SOSTDC1 expression levels might be associated with patient outcomes. To this end, we assessed SOSTDC1 levels in publically available databases of gene expression and survival in breast cancer patients. We assembled a super cohort comprised of six independent breast cancer cohorts totaling 741 individual breast cancer cases and their accompanying Affymetrix microarray and survival data (Supplemental Table 1). Quartile analysis revealed that approximately 75% of breast cancer cases exhibit comparably low levels of SOSTDC1 expression; however, in the upper quartile of expression, we observed a substantial shift in the overall distribution, with tumors displaying highly elevated SOSTDC1 levels (Fig. 2a). We asked whether SOSTDC1 expression levels are associated with patient distant metastasis-free survival (DMFS). Kaplan–Meier analysis of patients grouped by quartile revealed that patients with the highest quartile of SOSTDC1 expression exhibited significantly improved DMFS ($P = 0.025$) as compared to the lower three quartiles. Metastasis-free survival of patients in the lower three quartiles of SOSTDC1 expression did not differ significantly from one another (Fig. 2b). These findings point to a potential protective effect for SOSTDC1 overexpression in primary human breast cancer.

SOSTDC1 protein levels decrease as tumor size and stage increase

To extend these findings to SOSTDC1 protein levels, breast cancer tissue microarray (TMA) sections were immunostained for SOSTDC1. These TMAs contained formalin-fixed, paraffin-embedded samples from 81 breast tumors and five normal breast tissues, including three matched pairs. The TMA samples have been correlated with multiple clinical parameters, permitting the SOSTDC1 staining levels to be correlated with tumor parameters and patient outcome. SOSTDC1 staining associated significantly with tumor size, with larger tumors having less SOSTDC1 than smaller tumors ($P = 0.011$, Table 1). SOSTDC1 levels also correlated significantly with patient stage, with SOSTDC1 levels decreasing as patient stage increased ($P = 0.02$). Although there was a trend toward decreasing SOSTDC1 with increasing tumor grade, the decrease was not statistically significant (Table 1). Other parameters, including patient ethnicity and hormone receptor status, did not vary significantly with SOSTDC1 expression (data not shown).

SOSTDC1 secretion is reduced in breast cancer cells relative to normal mammary epithelial cells

After observing that SOSTDC1 message and protein levels were lower in breast tumors, we turned to cell lines to investigate how secreted and cellular SOSTDC1 varied with transformation. For this analysis, we used a model of breast cell stepwise transformation, along with two well-characterized breast cancer cell lines. In the stepwise transformation model, human mammary epithelial cells are progressively transformed with stable expression of telomerase, Simian virus 40 (SV40) small and large T antigen, and H-ras [36]. As shown in Fig. 3, cytosolic SOSTDC1 levels were reduced and no secreted SOSTDC1 was detected in either of the immortalized breast cancer cell lines, MCF7 and T47D (Fig. 3, lanes 7 and 8). In contrast, in the stepwise transformation model, SOSTDC1 levels from cell

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lysate samples remained steady despite varying stages of transformation. Nevertheless, SOSTDC1 secretion was reduced in cells transformed with SV40 and H-ras (Fig. 3, lanes 4–6) relative to normal or human telomerase (hTERT) immortalized cells (lanes 2 and 3). We also noted distinct patterns of banding within the media samples. The reason for this is unclear, although it may be attributable to the presence secreted SOSTDC1 with varying levels of glycosylation [44]. These findings suggest that SOSTDC1 secretion is reduced in breast cancer cells by two separate mechanisms, one involving decreased transcription and one involving decreased secretion.

Wise modestly enhances Wnt3a-induced beta-catenin activation in MDA-MB-231 cells

SOSTDC1 modulates both BMP- and Wnt-induced cell signaling [16], either increasing or decreasing Wnt-induced signaling, depending on the context [11, 28]. To test how SOSTDC1 affects Wnt-induced signaling in breast cancer cells, MDA-MB-231 cells were cotreated with Wise-containing conditioned media in the presence or absence of Wnt3a. For these experiments, conditioned medium from cells transfected to express secreted Wise was used, as previous reports have shown that human SOSTDC1 and its orthologue, Wise, function comparably in vitro [29, 44]. As expected, Wnt3a treatment led to the stabilization of active beta-catenin, which is indicative of the ligand's activation of the canonical Wnt signaling pathway (Fig. 4; compare lane 1 to lane 2). This activation was blocked by cotreatment of the known Wnt signaling antagonist, DKK1 (Fig. 4; compare lanes 2 and 3). In contrast, when cells were treated with Wise and Wnt3a concomitantly, the beta-catenin activation was not diminished (Fig. 4; lanes 1–4). Instead, this treatment led to mildly increased beta-catenin stabilization (179 ± 46% of control, $n = 3$), similar to previous reports [29].

Wise prevents BMP-7-induced Smad phosphorylation without affecting BMP-2-mediated Smad activation in MDA-MB-231 and MCF7 cells

To test how Wise treatment regulates BMP signaling in breast cancer cells, serum-starved MDA-MB-231 cells were cotreated with Wise-containing conditioned media and either BMP-2 or BMP-7. Simultaneous treatment with the known BMP inhibitor, Noggin, was included as a control. As expected, treatment of serum-starved cells with either BMP-2 or BMP-7 led to activation of Smad signaling, as evidenced by receptor Smad phosphorylation (Fig. 5, compare lanes 1 to 2 and 5). This activation was antagonized by cotreatment with Noggin (Fig. 5, compare lanes 2–3 and 5–6). When cells were treated with BMP-2 and Wise, however, BMP-induced Smad phosphorylation was not blocked (Fig. 5, compare lanes 3 and 4). In contrast, Wise strongly inhibited BMP-7-mediated activation of Smad phosphorylation (Fig. 5, compare lanes 6 and 7).

An inhibitory effect of BMP-7-induced Smad phosphorylation was also observed with recombinant human SOSTDC1 protein and an additional breast cancer cell line, MCF7. Smad phosphorylation induced by BMP-7 was blocked when cells were treated with SOSTDC1 (Fig. 6). As observed in the MDA-MB-231 cells, recombinant human SOSTDC1, like the Wise orthologue, did not block BMP-2-induced Smad phosphorylation in MCF7 cells (Fig. S1). As expected, cotreatment of the control BMP antagonist Noggin also reduced receptor Smad phosphorylation following either BMP-2 or BMP-7 treatment. Together, these results show that SOSTDC1 differentially affects signaling induced by BMP-2 and BMP-7 in breast cancer cells: SOSTDC1 is unable to abrogate activation by BMP-2, but acts as BMP-7 antagonist.

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Discussion

The results of this study demonstrate that SOSTDC1, a secreted regulator of two signaling pathways, is underexpressed in breast cancer and that it can differentially affect signaling induced by Wnt3a, BMP-2, and BMP-7. In breast cancer cells, SOSTDC1 modestly increases Wnt3a signaling, decreases BMP-7 signaling, and does not affect BMP-2-induced signaling. It follows that attenuated expression of SOSTDC1 in breast cancer might exert opposing effects on Wnt and BMP pathways, decreasing Wnt3a signaling while increasing BMP-7 signaling.

These effects may influence the clinical behavior of breast cancer. We observed that SOSTDC1 mRNA levels were reduced in breast tumors compared to normal breast tissue (Fig. 1). Analysis of microarray data from a combined cohort of 741 primary breast cancer cases further confirmed that SOSTDC1 expression is relatively low in the majority of breast cancer cases, but that a fraction of breast tumors (~25%) show markedly elevated SOSTDC1 levels (Fig. 2a). Primary tumors with the highest levels of SOSTDC1 corresponded significantly with improved distant metastasis-free survival compared to low expressors (Fig. 2b), suggesting that SOSTDC1 overexpression in the primary breast tumor confers a survival advantage to breast cancer patients. Our analysis of SOSTDC1 protein expression similarly demonstrated that reduced SOSTDC1 protein levels correlate with increased tumor size and stage (Table 1).

Our results demonstrating reduced SOSTDC1 mRNA levels in patient samples (Fig. 1) and reduced SOSTDC1 secretion in breast cancer cell lines (Fig. 3) suggest that two mechanisms, one transcriptional and one secretory, may regulate SOSTDC1 in breast cancer cells. Although inhibitors and effectors of *SOSTDC1* transcription have yet to be studied in the context of cancer, transcriptional control of *SOSTDC1* by the HOXA13 regulator is known to have developmental effects [46]. Additionally, examples of secretory control of SOSTDC1 and the Wnt pathway are abundant. For example, Wise, a SOSTDC1 orthologue, can undergo retention within the endoplasmic reticulum, where it can interact with the LRP6 receptor to block Wnt-induced signaling [29]. In contrast, secreted Wise can enhance or diminish Wnt signaling in a ligand-dependent manner [29]. It follows that inefficient exit of Wise from the ER would strongly impact its function. Another possible mechanism of SOSTDC1 action could be through ER trapping. For example, another Wnt inhibitor, Shisa, exerts its effect on signaling from within the ER, binding the Frizzled in the ER and preventing it from reaching the cell surface [47]. Likewise, Wnt ligands can themselves be trapped within the ER, thereby inhibiting signaling [48]. Future studies to elucidate the contributions of transcriptional and secretory SOSTDC1 regulation will expand our understanding of the mechanisms of SOSTDC1 reductions.

Roles for BMPs in cancer are complex and situational [49]; however, our observations that SOSTDC1 expression is decreased in breast cancer and that it inhibits BMP-7-induced signaling are consistent with reports on the role of BMP-7 in breast cancer. Several studies have demonstrated that BMP-7 is overexpressed in breast tumors relative to breast tissue [29, 32, 44]. This overexpression has been shown to promote the development of early bone metastases [45]. Breast cancer cell lines have been shown to have varied levels of BMP-7 and distinct responses to its removal or administration; however, BMP-7 administration to MDA-MB-231 breast cancer cells evokes increases in cell survival, migration, and invasion [46]. Thus, reduced SOSTDC1 expression may contribute to breast tumorigenesis by promoting BMP-7-dependent signaling.

Our observation that SOSTDC1 does not affect BMP-2 signaling in breast cancer suggests that the responses of SOSTDC1 to BMP ligands are tightly controlled in breast tissue.

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BMP-2 has anti-proliferative effects on breast epithelial cells at low concentrations, affecting cell cycle proteins p21, retinoblastoma (Rb) protein, and the phosphatase and tensin homolog (PTEN) tumor suppressor [47,48]. BMP-2 can also counteract estrogen-induced proliferation of breast cancer cells [50]. The differential activity of SOSTDC1 on BMP-2 and BMP-7 signaling might be therapeutically advantageous: a therapeutic strategy involving restoration of SOSTDC1 activity might counteract the tumor-promoting effects of BMP-7 signaling without affecting the anti-proliferative effects of BMP-2.

Effects of SOSTDC1 on Wnt3a activity were modest, and tended to increase rather than decrease Wnt signaling (Fig. 4). The involvement of canonical Wnt signaling has been demonstrated in numerous cancer types [39–41]. Given that SOSTDC1 blocks Wnt-induced transcriptional activation in renal carcinoma cells [16], the observation that Wise treatment increases Wnt3a-mediated beta-catenin activation in breast cancer cells was somewhat surprising but not entirely unexpected, since secreted SOSTDC1 can either activate or inhibit Wnt signaling [29]. It is possible that another Wnt ligand may represent a more critical target of SOSTDC1 in breast cancer. Alternatively, the role of SOSTDC1-dependent control of Wnt signaling may be of secondary significance in breast cancer, particularly since the relevance of Wnt signaling in breast cancer has been less clear than in other cancer types [34, 42].

Normal cell growth involves a complex interplay between multiple signaling pathways. Effects of SOSTDC1 underexpression will depend on numerous factors. The delicate relationship between multiple cellular signals, along with SOSTDC1's differential regulation of Wnt3a and BMP-7 signaling, place SOSTDC1 in a position to have a distinct effect on cancer cell development, growth, or progression. How restoration of this protein will affect oncogenesis and progression remains to be seen; it may well vary with cancer profile and cellular environment. Future studies of SOSTDC1's role in breast cancer will address these issues, enhancing our understanding of this multifaceted disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
AUS	Antigen unmasking solution
bax	Bcl-2-associated X protein
BMP	Bone morphogenetic protein
CK1gamma	Casein kinase 1 gamma
DAB	Diaminobenzidine
DKK1	Dickkopf-1

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DMEM	Dulbecco's Modified Eagle's Medium
DMFS	Distant Metastasis-Free Survival
ER	Endoplasmic reticulum
EsR	Estrogen Receptor
FBS	Fetal bovine serum
Fzd	Frizzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GSK-3	Glycogen synthase kinase-3
HME	Human mammary epithelial cells
HRP	Horseradish peroxidase
hTERT	Human telomerase
IgG	Immunoglobulin G
kDa	Kilodalton
LN	Lymph node
LRP	Low density lipoprotein receptor-related protein
MEGM	Mammary epithelial cell growth media
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma protein
SDS	Sodium dodecyl sulfate
SOSTDC1	Sclerostin domain containing 1
SV40	Simian virus 40
T	Tamoxifen
TMA	Tissue microarray
Wnt	Wingless/int

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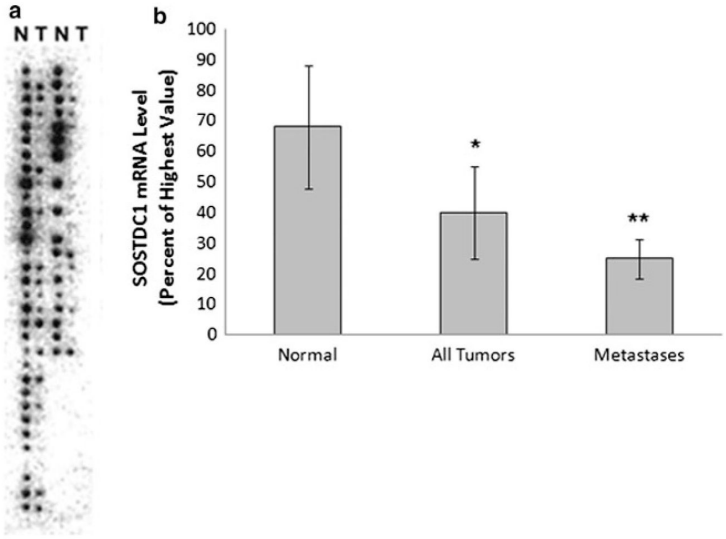

Fig. 1.

Analysis of SOSTDC1 message level in matched normal (N) and tumor (T) samples from breast cancer patients. **a** Samples were probed with radiolabelled SOSTDC1-specific probes. Tumor samples and patient-matched controls are positioned adjacently. **b** Signals were quantified using UnScanIt Image software. Normal samples ($n = 50$) showed an average signal of 67.6% of the maximum staining intensity. Average signal intensity of all tumor samples ($n = 53$) was 38.9% of the maximum intensity, and signal intensity of metastases ($n = 3$) was 25.4% of maximum. Average staining intensity of the tumors is approximately 60% of all normal tissue (* $P < 0.05$), while metastases show even less SOSTDC1 (** $P < 0.01$)

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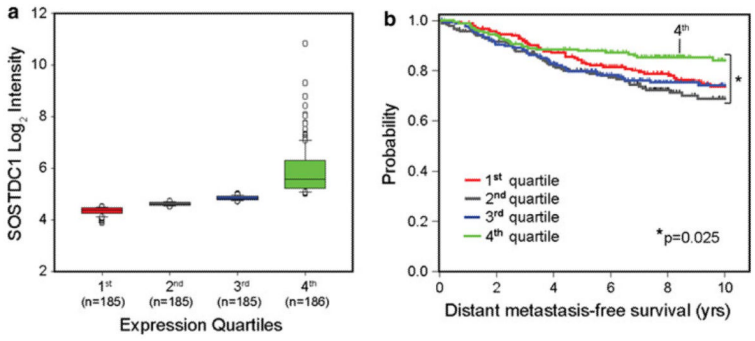

Fig. 2.

SOSTDC1 expression is associated with distant metastasis-free survival of breast cancer patients. **a** Breast cancer patients were grouped into quartiles according to the SOSTDC1 mRNA expression levels. Box and whisker plots reflecting the distribution of SOSTDC1 expression within each quartile is shown. **b** Kaplan–Meier survival curves were generated to compare the DMFS of patients between SOSTDC1 expression quartiles. The likelihood ratio test *P* value is shown

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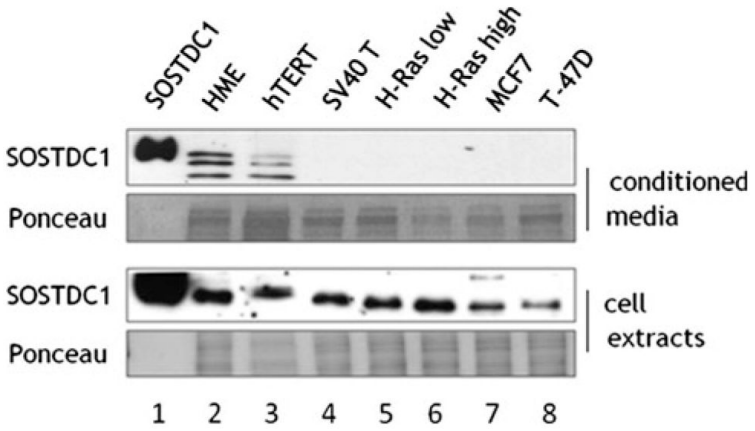

Fig. 3.

SOSTDC1 secretion is decreased in breast cancer cell lines. Protein from concentrated conditioned media and that from cell extracts was immunoblotted for SOSTDC1. Recombinant SOSTDC1 is used as a positive control. Cell lines analyzed include cells in a model of breast cancer progression [45]: human mammary epithelial cells (HME); HMEs stably overexpressing human telomerase (hTERT); hTERT cells stably expressing the large and small SV40 T antigens (SV40 T); SV40 T cells stably expressing H-ras V21 at low and high levels (H-ras V21 low and H-ras V21 high); and MCF7 and T-47D breast cancer cells

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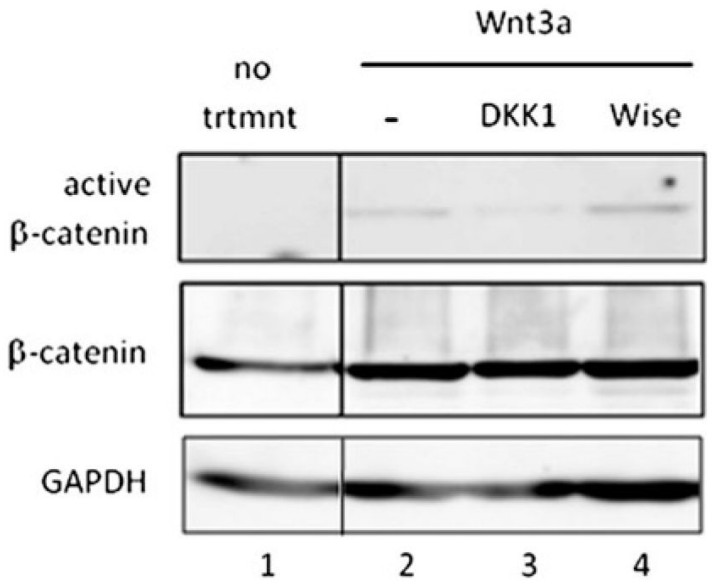

Fig. 4.

SOSTDC1 orthologue, Wise, modestly increases Wnt3a-induced beta-catenin activation. Serum-starved MDA-MB-231 cells were treated with Wnt3a (25 ng/ml) for 2 h in the presence or absence of DKK1 (100 ng/ml) or Wise-containing conditioned media. Equal amounts of cellular proteins from each treatment group were gel-resolved and immunoblotted with antibodies specific for active beta-catenin, inactive beta-catenin, and GAPDH

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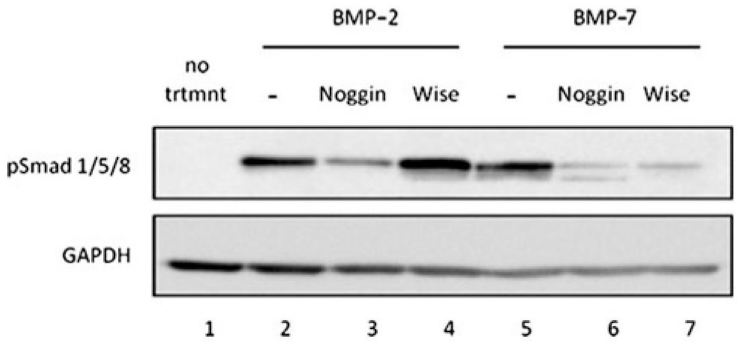

Fig. 5.

SOSTDC1 orthologue, Wise, prevents BMP-7-induced Smad phosphorylation without affecting BMP-2-mediated Smad activation. Serum-starved MDA-MB-231 cells were treated with BMP-2 or BMP-7 (100 ng/ml) for 2 h in the presence or absence of Noggin (100 ng/ml) or Wise-containing conditioned media. Equal amounts of cellular proteins from each treatment group were gel-resolved and immunoblotted with antibodies specific for phosphorylated Smad proteins and GAPDH

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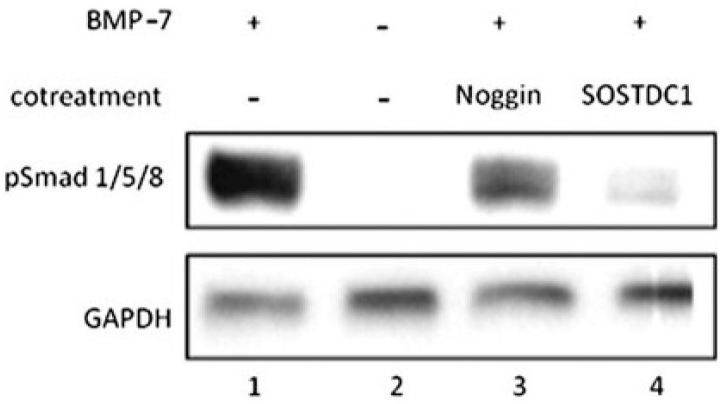

Fig. 6.

SOSTDC1 inhibits BMP-7-induced Smad phosphorylation in breast cancer cells. MCF7 cells were treated for 4 h with BMP-7 (75 ng/ml) in the presence or absence of Noggin or SOSTDC1 (150 ng/ml). Cellular proteins were immunoblotted for phosphoSmads 1/5/8. GAPDH was used as a loading control

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Table 1

SOSTDC1 protein levels decrease as tumor size and stage increase

Correlations between SOSTDC1 and tumor parameters								
Tumor size mm^3	**Mean SOSTDC1 (SD)**	***n***	**Stage**	**Mean SOSTDC1 (SD)**	***n***	**Grade**	**Mean SOSTDC1 (SD)**	***n***
0 to <20	35.7 (14.7)	14	1	38.3 (13.1)	12	1	39.5 (13.8)	6
20 to <50	34.1 (13.2)	42	2	33.2 (14.3)	48	2	31.0 (13.8)	19
>50	23.3 (15.9)	15	3	24.6 (12.5)	13	3	29.4 (15.1)	39
Correlation coefficient (for continuous data) = −0.30 *P* = 0.011			4	37.8 (NA)	1		Correlation coefficient (for continuous data) = −0.14 *P* = 0.28	
				Correlation coefficient (for continuous data) = −0.27 *P* = 0.02				

SOSTDC1 protein levels were assessed by immunohistochemistry in 81 breast tumor samples within tissue microarrays and quantified by measurement of pixel intensities, as previously described [43]. Mean intensities are reported in arbitrarty units, with standard deviations shown in parentheses